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LOCALIZATION OF CARBOHYDRASES AT THE SURFACE OF FUNGUS SPORES BY ACID TREATMENT

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THE localization of enzymes at the surface of cells has been reported by several groups of investigators (for literature citations see 1, 3, 8, 11). In all cases the nature of the evidence employed has been indirect, with the possible exception of phosphatase demonstrated by the Gomori technique. While this method is extremely useful in many situations, its value for localization at the cell surface is limited due to the extreme thinness of the plasma membrane. Thus it cannot be used to distinguish between a locus at the external surface of the membrane and one interior to the membrane.

The lack of direct evidence for surface location increases the value of different types of indirect data. A potentially useful technique for certain types of cells is based on the phenomenon of inactivation of certain enzymes of intact cells by exposure to low pH without disruption of cellular metabolism. Many years ago it was known that treatment of yeast cells with acid destroyed their invertase activity, yet did not kill the cells nor interfere seriously with their fermenting properties (16). This evidence was used to support the hypothesis of direct fermentation of sugars, yet it can also serve to localize invertase at the surface of the yeast cells as was subsequently claimed by Wilkes and Palmer (15) and Nelson and Wilkes (10) on the basis of similarities of the pH responses and kinetics of the enzyme *in vitro* and *in vivo*. In a previous paper (8) acid inactivation of an enzyme, in intact *Myrothecium verrucaria* spores, which oxidizes ascorbic acid was reported. These data, together with results of other experiments showing the similarities of the pH activity curves for the enzyme, were used to localize the enzyme at the cell surface. While it was stated that the acid treatment did not affect the viability nor the respiratory activity of the spores, such data were not reported. The present paper reports the results of such experiments. In addition the inactivation of several carbohydrases in spores of *M. verrucaria* and *Aspergillus luchuensis* by acid treatment is demon-

strated. The relation of this type of evidence to the location of the inactivated enzymes is discussed and the conclusion reached that these enzymes are surface located.

METHODS

Spores of *Myrothecium verrucaria* (QM 460) were obtained from cultures grown as described previously (7). Spores of *Aspergillus luchuensis* (QM 873) were grown in similar manner with the exception of using 2 per cent sucrose as carbon source in place of the filter paper. To prepare spore suspensions of *A. luchuensis*, the spores were first wetted with distilled water containing 0.05 per cent Duponal. No wetting agent is necessary to prepare suspensions of *M. verrucaria* spores. In all cases the spores were washed three times by centrifugation and suspended in distilled water.

Acid treatment of the spores was effected by combining equal volumes of spore suspension with 0.2 N HCl and incubating for the indicated time at 30° C on a shaker. The acid treatment was terminated by either centrifuging the spores and washing with 0.05 M $KHPO_4$ buffer at pH 6 or by adding an aliquot of K_3PO_4 known to bring the acid solution to about pH 6. Hydrochloric acid was used in all of the experiments reported here although sulfuric acid works equally well, at least for *M. verrucaria* spores. While nitric acid inactivates the invertase of these spores it also kills the cells.

Carbohydrase activity was measured by adding the appropriate substrate (1 per cent final concentration) to spore suspensions and incubating on a shaker at 30° C. Hydrolysis of non-reducing substrates was followed by determination of reducing sugars at appropriate intervals by the dinitrosalicylic acid method of Sumner (13), and of reducing substrates by determination of glucose with the glucose oxidase method of Keilin and Hartree (5, 6).

Respiration was measured by conventional Warburg technique. Viability was determined by diluting the suspensions and plating out on agar containing sucrose and yeast extract. In all cases where viability determinations were carried out, aseptic precautions were observed. Growth was measured by cell volume determinations in hematocrit tubes after incubation with sugar and yeast extract (9).

RESULTS

Inactivation of the invertase of *M. verrucaria* spores by acid treatment is shown in Fig. 1 a. After continued incubation with sucrose the acid treated spores commence to hydrolize the sucrose, the rate increasing with time. If, however, toluene is added to kill the acid treated cells no resynthesis or reactivation of the invertase occurs. The development of invertase activity after acid inactivation represents resynthesis of the enzyme rather than reactivation after restoration to solutions of normal pH. This point will be discussed in more detail further on.

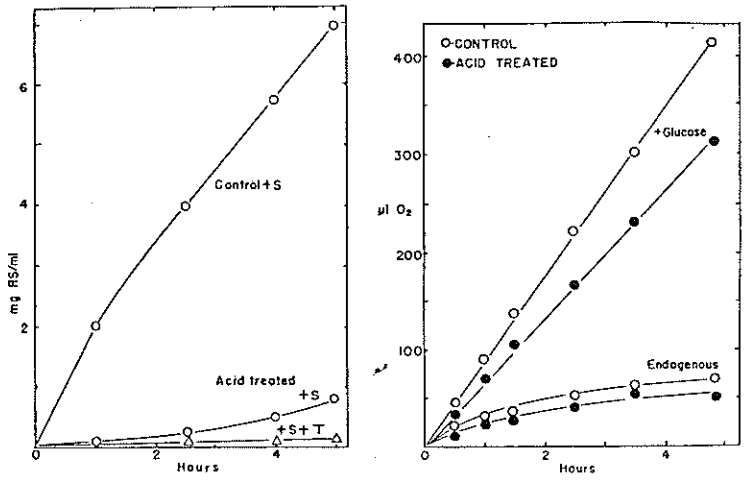

Fig. 1 A. Fig. 1 B.

Fig. 1. Effect of acid treatment on (A) invertase and (B) respiration of *M. verrucaria* spores (20 mins. acid treatment; acid removed by washing; in (A) S = sucrose, T = toluene).

Of critical importance in interpreting this phenomenon of acid inactivation is the effect of the treatment on other physiological processes of the cells. Data show (Fig. 1 b) that neither the exogenous (with glucose) nor endogenous respiration of the spores is decreased greatly. Other data demonstrate that acid treated spores which cannot hydrolyze sucrose respire this sugar at a rate comparable to that for controls. This is ascribed to non-hydrolytic metabolism of sucrose, as will be brought out in a subsequent paper.

To test further the effect of acid treatment on the spores, viability tests were carried out as well as measurements of capacity for growth. Previous studies have demonstrated that spores of *M. verrucaria* swell rapidly in solutions of yeast extract and sucrose, and that the rate of swelling can be used as a measure of growth rate. This swelling is a true growth phenomenon, being accompanied by more or less parallel increases in dry weight and by germination (9). Measurements of invertase activity were run concurrently. The data show (Table I) that while one minute exposure to 0.1 N HCl inactivates almost completely the invertase of the spores, there is no significant effect upon increase in cell volume even after 25 minutes exposure. While the viability appears to have been affected to an appreciable extent by this treatment, the significance of the differences is un-

TABLE I

Effect of acid treatment on invertase activity, viability and rate of growth of *Myrothecium verrucaria* spores.

(Acid neutralized with K_3PO_4).

	Duration of acid treatment	0	1	2	3 hrs.
Invertase activity ..	0 mins.	0	0.20	0.39	0.60 mg R.S./ml
	1 »	0	0	0.10	0.15 » »
	5 »	0	0	0	0 » »
	25 »	0	0	0	0 » »
Cell volume	0 mins.	16	26	41	89 µl
	1 »	16	24	36	82 »
	5 »	16	24	36	82 »
	25 »	16	23	31	84 »
Viability	0 mins.	1.6×10^8 viable spores/ml of susp'n.			
	1 »	1.3 » » » » » » »			
	5 »	1.3 » » » » » » »			
	25 »	1.0 » » » » » » »			

certain. Acid treatment affects the surface properties of the spores. The decrease in viability is considered to be apparent and may be ascribed to some clumping of the treated spores or to their adhesion to the walls of flasks and pipettes during dilution to appropriate concentrations for plating out. The cell volume assay is considered a more reliable measure of viability in this case since the increase in cell volume is a function of the number of viable cells present, and constitutes better evidence attesting to lack of injury to vital metabolic pathways within the cells.

More ideal carbohydrase systems to localize at the spore surface by the acid treatment technique would be the amylases or the cellulases. In these cases the large molecular size of the substrate would in itself tend to exclude penetration into the cell for hydrolytic cleavage. Unfortunately suspensions of intact, viable spores of *M. verrucaria* do not hydrolyze these substrates (starch or cellulose) at measurable rates.

Studies of the metabolism of carbohydrates by *A. luchuensis* spores have shown that they hydrolyze sucrose and cellobiose at rates greatly in excess of their metabolic requirements; maltose and trehalose are hydrolyzed slightly in excess (unpublished). After acid treatment these spores no longer

TABLE II

Effect of acid treatment on hydrolysis of sugars by *Aspergillus luchuensis* spores.

(20′ acid treatment; acid removed by washing; data for sucrose in terms of red. sugar, for other sugars as glucose—all in γ/mg spores/hr.).

Sugar	Treatment	Rate of hydrolysis
Sucrose	Control	116
	Acid treated	7
Maltose	Control	40
	Acid treated	0[1]
Trehalose	Control	24
	Acid treated	0[1]

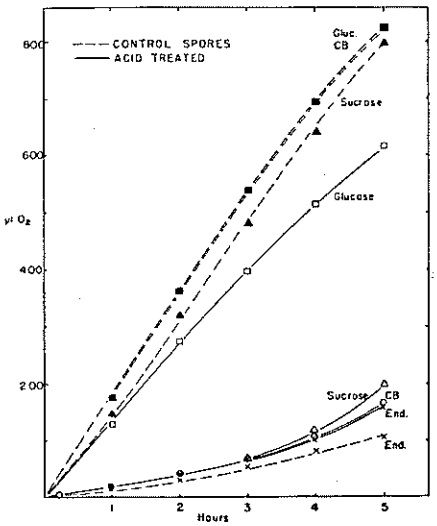

Fig. 2. Effect of acid treatment on endogenous (End.) respiration of *A. luchuensis* spores and on respiration with glucose (Gluc.), cellobiose (CB), or sucrose.

hydrolyze sucrose, maltose or trehalose (Table II). Furthermore, such spores cannot respire sucrose, yet respiration on glucose and endogenous respiration is only slightly affected (Fig. 2). While the cellobiase activity of acid treated spores has not been measured, the inability of such spores to respire cellobiose (Fig. 2) indicates cellobiase inactivation.

[1] No measurable hydrolysis indicates less than 4 γ/mg/hr.

DISCUSSION

In considering the results of this study several basic facts should be pointed out. The enzymes studied here are all present in excess of the metabolic requirements of the cells for the products of enzymatic action. Thus enzymatic activity of the living spores can be measured by analysis of the suspension medium. It is also fundamental that these enzymes are not released from intact cells.

Data presented here have demonstrated the inactivation of the invertase of *M. verrucaria* spores and also of the invertase, cellobiase, trehalase and maltase of *A. luchuensis* spores by brief exposure of the spores to acid. Interpretation of the significance of these data hinges upon a knowledge of what other effects such treatment has upon the cells. Of primary importance in this connection is the establishment of the resistance of the spores used to the acid. Exposure for periods of time greatly in excess of that required for inactivation of the enzymes in question has no appreciable effect upon the rate of germination (as measured by cell volume increase), nor are significant numbers of the spores killed. Furthermore, the respiratory activity of the spores is not greatly impaired except where the substrate is metabolized through the inactivated enzyme. On the basis of this information it would not seem possible that hydrogen ions penetrate the cell to inactivate certain enzymes within the cell. If such were the case, a more fundamental disruption of metabolism should occur. Those enzymes which are inactivated must therefore be at the external surface of the plasma membrane. In a previous study the surface location of invertase in *M. verrucaria* spores was inferred from the pH relation of the enzyme *in vitro* and *in vivo*.

The mechanism of inactivation of the enzymes is unknown. Some unpublished data show, however, that the effect of acid on the cells is not restricted to a simple denaturation of protein. Thus analyses of the acid solution after treatment of the spores show phosphates and nitrogen. Furthermore some carbohydrate would appear to be split off from the cells since autolysis of acid treated spores under toluene results in considerably less production of reducing sugars than controls. It is perhaps significant that removal of the acid by washing the spores or by neutralization yields the same results. In none of the cases studied is the inactivation readily reversible as Herriott (4) found for invertase extracted from yeast. Restoration of activity does occur in cells incubated with sucrose overnight (more

rapidly if yeast extract is added). If the cells are killed by adding toluene or if they are suspended only in buffer, nutrient salts solution, or distilled water, invertase activity does not reappear. Furthermore, the "reactivation" does not occur anaerobically nor at temperatures beyond the growth limits of the organism. Hence it is concluded that the development of invertase activity after acid inactivation is due to resynthesis of the enzyme. Whether this represents total synthesis or merely repair of a critical portion of the enzyme is not known. Certainly further experimentation with this type of material could provide valuable data on the problem of enzyme synthesis.

It is interesting to consider in more detail the possible implications of the localization of enzymes at the cell surface upon our concept of the nature of the plasma membrane and also to evaluate more precisely the meaning of cell surface as the locus of enzymic activity. Davson and Danielli (7) conceive of the plasma membrane as consisting essentially of a double film of lipoid molecules bounded on either side by a proteinaceous layer. In the past our thinking has limited the function of this membrane to its properties of differential permeability which regulate, in a physical manner, the diffusive interchange of materials between the interior of the cell and its environment. The localization of enzymes at the cell surface, the biochemical participation of the membrane in the penetration of phosphate into the cell by formation of an organic phosphate which enters the cell, the suggested phosphorylation of sugars at the membrane, the high degree of specificity for penetration of the erythrocyte by sugars, all point toward an active biochemical role of the plasma membrane in addition to its functions of controlling diffusion. This has been discussed in detail by Rosenberg and Wilbrandt (11) and also by Rothstein *et al.* (17). The protein of the plasma membrane then functions not only by endowing the membrane with certain structural properties, but has an additional, more active role in governing the metabolism of the cell. If we were to accept Virtanen's suggestion that all of the protein of the cell is enzymatic in nature (14), then all of the protein of the plasma membrane has enzymatic properties. Certainly the number and variety of types of surface located enzymes is becoming ever greater.

SUMMARY

1. Treatment of spores of the fungi *M. verrucaria* or *A. luchuensis* with 0.1 N HCl destroys the enzymatic activity of the spores toward certain

substrates yet does not significantly affect the viability of the cells, their subsequent rate of growth, nor their general metabolic activity.

2. It is concluded that the enzymes inactivated by this treatment are at the external surface of the plasma membrane. Such enzymes in *M. verrucaria* spores are invertase, and in *A. luchuensis* spores, invertase, cellobiase, trehalase, and maltase.

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